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Study on the laminar hydathodes of *Ficus formosana* (Moraceae) V.: Divergent evolution between stomata and water pores

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ABSTRACT: Water pores and stomata play roles in water regulation through guttation and transpiration, respectively. On the *Ficus formosana* leaves, water pores are present in the hydathodes on the upper surface, whereas stomata are randomly distributed on the abaxial epidermis of non-vein regions. Here, we investigate the development and physiological functions of water pores and stomata from the same leaves and explore their evolutionary relationships. We compare their structures using optical and electron microscope, and establish their functions through physiological experiments. *Ficus formosana* Maxim. f. *shimadae* Hayata water pores are almost circular, whereas its stomata are elliptical. Water pores are clustered and occur at a higher density than stomata, with these latter being anomocytic. Our ultrastructural analysis shows that *F. formosana* f. *shimadae* water pores contain amyloplasts and have thickened walls around the pores, with many plasmodesmata observed during their development. The chloroplasts of the stomatal guard cells possess typical plant cell grana and thylakoids, and the inner walls around the stomatal space are thickened. The differentiation and developmental processes of water pores and stomata are similar. Stomatal apertures were regulated by light/dark, fusicoccin, ABA, or mannitol treatments, but water pores were not. Our findings indicate that water pores and stomata on the *F. formosana* f. *shimadae* leaves evolved divergently.

KEY WORDS: Divergent evolution, *Ficus formosana*, laminar hydathodes, Moraceae, Stomata, Water pores.

INTRODUCTION

Guttation is the process by which water in liquid form is exuded through the water pores of plant hydathodes, and transpiration involves evaporation of water as vapour through stomata on leaf surfaces (Esau, 1977). Guttation is a passive phenomenon, with excess liquid exudation at leaf vein-ends primarily mediated by root pressure (Taiz and Zeiger, 1991; Salisbury and Ross, 1992). Water pores resemble oversized stomatal structures, the pore apertures of which are not regulated and remain permanently open in many plants (Mortlock, 1951; Lersten and Curtis, 1985, 1986, 1991; Donnelly and Skelton, 1987; Perdersen *et al.*, 1997; Martin and von Willert 2000). Recently, Wightman *et al.* (2017) reported that *Saxifragga cochlearis* develops a hydathode pit, differentiated from guard-type cells and creating a thickened rim encircling the hydathode pore, and it is distinctly different from other epithemal hydathodes. In addition, bacteria and fungal hyphae are often found in the vicinity of hydathodes following long-term guttation (Maeda and Maeda, 1988; Chen and Chen, 2005). Both water pores and stomata are the natural pathways for bacterial and fungal infection of plant leaves, and it has been proposed that certain innate immune mechanisms are involved in plant-pathogen coevolution (Hugouvieux *et al.*, 1998; Grunwald *et al.*, 2003; Melotto *et al.*, 2008; Zeng *et al.*, 2010; Cerutti *et al.*, 2017). Unlike water pores, stomata actively regulate water transpiration and gas exchange by modulating the size of the aperture via their two flanking guard cells that respond to environmental (abiotic and biotic) factors such as light intensity, CO_2 concentration, drought, UV-B light, relative air humidity, ozone levels, and pathogen infection (Roelfsema and Hedrich, 2005; Melotto *et al.*, 2006; Casson and Gray, 2008; Assmann and Jegla, 2016; Panchal *et al.*, 2016). Several ion pumps, transporters, and channels associated with H^+, K^+, Ca^{2+} and anions in the plasma membrane are involved in how guard cells control stomatal apertures (Kollist *et al.*, 2014; Murata *et al.*, 2015).

Most of the available literature concerning hydathodes structure present light or electron microscopy studies (Maeda and Maeda, 1987; 1988). However, a comparative analysis of the evolutionary relationships between water pores and stomata is lacking, especially with regard to their thickened wall structures. Previous studies in our laboratory focused on the external features, internal structure and morphogenesis of *Ficus formosana* Maxim. f. *shimadae* hydathodes, as well as the impact of salt injury on guttation (Chen and Chen, 2005; 2006; 2007). Here, we use anatomical and ontogenetic analyses, as well as plant physiology experiments, on water pores and stomata of the *F. formosana* f. *shimadae* leaves to evaluate their evolutionary relationships.

MATERIALS AND METHODS

Plant material

Plants of *Ficus formosana* Maxim. f. *shimadae* Hayata were planted in soil pots within a greenhouse of

the Department of Botany, National Taiwan University. An automatic device was used for daily watering and fertilizer supplementation, as well as for temperature and humidity recording, throughout the experimental period, as described (Chen and Chen, 2005).

Light microscopy (LM)

Under a dissecting microscope, leaf samples containing achlorophyll hydathodes and hypodermal stomata at different stages were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 4 h at room temperature and then washed in a rinse buffer of 0.1 M sodium cacodylate. These samples were then fixed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.0) for 4 h, before being washed in 0.1 M sodium cacodylate buffer, dehydrated though a gradient of acetone and embedded in Spurr's resin (Spurr, 1969). Resin-embedded samples were polymerized at 70 °C for 8 h in an oven. The resulting plastic blocks were trimmed and cut using a Reichard ultramicrotome with a glass or diamond-coated blade. Sections (1 mm thick) were collected from the ultramicrotome using a glass blade. Upon full extension on a hot-plate at 60 °C, sections were stained with 0.1% Toluidine blue-0 for 2-3 min, excess dye was drained off, and then sections were air-dried and photographed with a Zeiss Photomicroscope III.

Scanning electron microscopy (SEM)

Sample fixation and dehydration are as described for LM above. After dehydration in the acetone series, samples were critical-point-dried by a critical point dryer (HITACHI Critical Point Dryer HCP-2), then were mounted on aluminum stabs with silver paste and ion sputter-coated with gold (Eiko Engineering, Ltd. IB-2 ion coater). Finally, the samples were d viewed in a Hitachi S-520 scanning microscope, and photographed.

Transmission electron microscopy (TEM)

Ultrathin sections (90 nm thick) were cut using a diamond-coated knife and transferred to a 75 mesh mounted on formvar film-supported grids. The section-mounted grids were stained with saturated aqueous uranyl acetate for 25 min, followed by lead citrate for 5 min. The stained sections were observed and photographed using a Hitachi H-600 transmission electron microscope at 75 kV.

Preparation of epidermal strips and physiological experimentation

To aid our investigation of aperture control by water pores and stomata, we isolated *F. formosana* f. *shimadae* epidermal strips containing water pores or stomata. Preparation of epidermal strips and experimental treatments were performed as described by McAinsh *et al.* (1996), with slight modifications. Isolated epidermal strips containing water pores or stomata (approx. 0.5 x 0.5 cm) were carefully peeled from adaxial hydathodes or abaxial leaf surfaces, respectively. Strips were floated on buffer solution (10 mM MES, 30 mM KCl, 0.1 mM $CaCl_2$, pH 6.1) for 10 min, before being overnight in the dark, and then transferred to treatment solutions. Epidermal strips were treated under light/dark conditions, or with applications of fusicoccin, ABA, or mannitol. For the light/dark treatment, epidermal strips were floated on buffer solution either under darkness (as a control) or under white light of a photon flux density, 120 μmol m^{-2} s^{-1} for 1 h. For fusicoccin treatment, epidermal strips were transferred to the buffer solution containing an additional 1 μM fusicoccin for 1 h under darkness. For ABA or mannitol treatment, epidermal strips were floated on buffer solutions containing either 10 μM ABA or 0.75 M mannitol for 1 h under white light, respectively, with strips in buffer lacking ABA or mannitol under white light acting as controls. All treated samples were fixed with 8% paraformaldehyde in buffer solution for 1 h and then washed three times in water, each time for 10 min. After washing, samples were stained with propidium iodide solution (PI, 20 ng/ml; Molecular Probes, Carlsbad, CA, USA) for 10 min, before again washing three times in water (each time for 10 min). Epidermal strips were observed using a confocal microscope (Zeiss LSM 510 META), and images were obtained using excitation/emission maxima of 535/617 nm for PI fluorescence and 365/405 nm for cell wall autofluorescence (Berg, 2004).

Measurement of stomatal apertures and statistical analysis

Widths of 20 randomly selected stomata or water pores (and their respective apertures or pores) were measured at 400X magnification using an Olympus BM2 microscope fitted with an eyepiece micrometer. Each experiment comprised three replicates, with usually one (sometimes two) strips constituting a treatment. Aperture values are represented as mean ±SE of three replicates. We used Student *t* tests to assess significant differences between control and treated samples, with a significance level of $p < 0.05$.

RESULTS

Morphological comparison of F. formosana* f. *shimadae* water pores and stomata: epidermal characteristics, stomatal type, and distributions

Cross-sections of mature leaves revealed that *F. formosana* f. *shimadae* Hayata hydathodes are complex and consist of epithem cells, the sheath layer surrounding them and many pores present on the upper epidermis (Fig. 1A). The structures of water pores and stomata on leaf surfaces are presented in the paradermal sections shown in Figs. 1B and 1C. Distributions and properties of the stomata and water pores on leaf surfaces

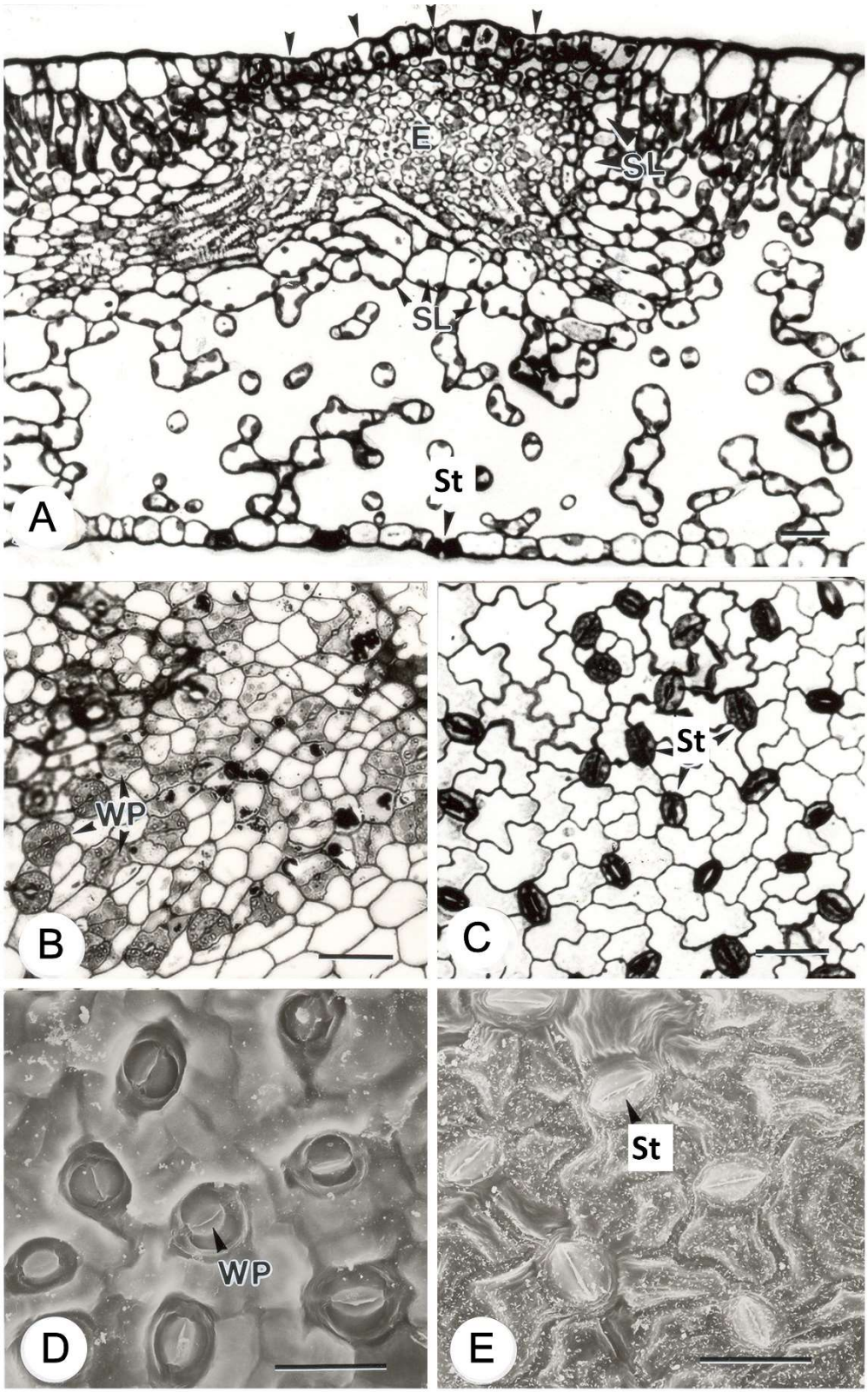

Fig. 1. Distribution and anatomy of water pores and stomata on the surface of ***F. formosana*** f ***shimadae*** leaves. **A**, Cross-section of laminar hydathodes. Arrowheads indicate epidermal water pores on the surface of hydathodes. **B**, Paradermal section of the epidermal layer of hydathodes. **C**, Paradermal section of hypodermal stomata. **D**, SEM image of water pore on the surface of hydathodes. **E**, SEM image of stomata. E, epithem; SL, sheath layer; St, stomata WP, water pore. Scale bars: **A-E**=20 μm.

Table 1. Cumulative data of stomata and water pore from ***Ficus formosana*** f. ***shimadae*** hydathodes.

Type	Length of paired cells (μm)	Width of paired cells (μm)	Length of pore (μm)	Ratio of pore length of cells length (μm)	Number[a] (10^4/ mm^2)
Stomata	19.63 ± 1.49	15.75 ± 1.61	11.58 ± 1.95	0.59 ± 0.03	8 ± 2
Water pore	18.83 ± 1.28	18.66 ± 1.44*	6.4 ± 0.82*	0.34 ± 0.02*	13 ± 2*

a. Based on counts from 50 samples; Stomatal counts based on vein-free regions, whereas counts of water pore are from hydathodes.
* Indicated significant difference between stomata and water pore (Student's test, $p < 0.05$).

are summarized in Table 1. Overall, water pores (represented by a pair of epidermal cells surrounding the aperture) are either circular or square, whereas stomata are elliptical. We found that water pores are significantly clustered in the hydathodes (Fig. 1B). The stomata exhibit a functional anomocytic unit, i.e., lacking prominent subsidiary cells (Fig. 1C). SEM analysis revealed that the water pores appeared in depressions on the surface of the hydathodes (Fig. 1D) and on the abaxial epidermis stomata are covered by a layer of cuticle consisting of two guard cells surrounding the apertures (Fig. 1E).

Ultrastructural observations of water pores and stomata

Ultrastructural analysis of stomata by means of TEM revealed thick outer and inner guard cell walls facing the stomatal chamber (Figs. 2A and 2C). The thick outer guard cell walls form slightly overlapping ridges can be observed in both young and mature leaves (Figs. 2A and 2B). We observed a noticeable thickening of the guard cell wall facing the stomatal chamber near the aperture (arrows in Fig. 2F), which was absent in the ventral wall along the long axis near the aperture (Figs. 2A-B and 2D-E). Mannitol treatment (0.75 M) induced plasmolysis in the cytoplasm (arrows in Fig. 2E) and almost completely closed stomatal apertures (Figs. 2E and 2F).

TEM revealed that the walls of the paired cells of water pores are also unevenly thickened, with the outer and inner walls thicker than the cell walls lining the interstitial space between the two peripheral cells (Figs. 3A-C). Pore length is less than one-third the length of the long axes of paired cells, with the overlapping ridges of the paired cells forming the aperture (Fig. 3A). Hydrostatic pressure upon the xylem reaching a threshold forces open these overlapping ridges, thereby facilitating guttation. Water pores remained open even after the leaf was treated with 0.75 M mannitol and caused cytoplasmic plasmolysis. (Fig. 3D).

Ontogenesis and pore formation of stomata and water pores

We assessed the ontogenetics of stomata and water pores using light microscopy and TEM. A stomatal lineage ground cell (SLGC) that may differentiate into pavement cells or may become meristemoid mother cells (MMCs, asterisk in Figs. 4A). Guard mother cells and other epidermal cells are formed via asymmetric division of precursor cells. Guard mother cells are smaller than typical epidermal cells, with these latter having a large central vacuole and cytoplasm restricted to peripheral regions (Figs. 4A and 4B). The volume ratio of nucleus to cell in guard mother cells is also much larger than that of epidermal cells. Chloroplasts, mitochondria and tiny vacuoles surround the active nucleus of guard mother cells. The substomatal intercellular space lies beneath the guard mother cells (Fig. 4C). Upon symmetric cell division, these guard mother cells form two elongated guard cells, in contact with each other along one of their thin inner walls (the middle lamella) (Figs. 4A, 4C and 4D). The rod-shaped nuclei of these young guard cells are centrally located, oriented parallel to the long axis of the elongated guard cells. The paired guard cells are characteristically kidney-shaped (Figs. 4C and 4D) and are surrounded by epidermal cells. They also possess prominent nuclei, many small vacuoles scattered throughout the cytoplasm, and exhibit an accumulation of aggregated starch granules in their chloroplasts. During stomatal pore formation, a highly electron-dense substance accumulates at the middle lamella (Figs. 4E and 4F). Starch-containing chloroplasts are present at all developmental stages of stomatal guard cells, with intact grana being observed at the mature stage (Figs. 4D and 4G). Radially-arranged microtubules were observed in the cytoplasm and associated with the thickened cell wall near the sub-stomatal cavity of paired guard cells (Fig. 4H).

The guard mother cells of water pores (indicated by asterisks in Figs. 5A and 5B) are also formed by asymmetric cell division of precursor cells. Upon cell enlargement, these guard mother cells undergo symmetric cell division to produce two peripheral cells of similar size (Figs. 5C and 5D). We observed multiple plasmodesmata between the paired water pore cells and their neighboring cells at this stage (Figs. 5A-D). Unlike stomatal development (wherein the substomatal chamber is present), the peripheral water pore cells and their guard mother cell are always intimately in contact with the underlying parenchyma cells (Fig. 5C). As the water pore cell pair matures, the cell wall matrix between them lyses to form the pore (arrowheads in Figs. 6A-D). However, as for stomata, we observed unevenly thickened cell walls, especially for outer and inner wall layers but additionally of the ventral wall of the water pore cell pair (Figs. 6D-H). The cell wall becomes increasingly thick from the ventral side to the outer wall,

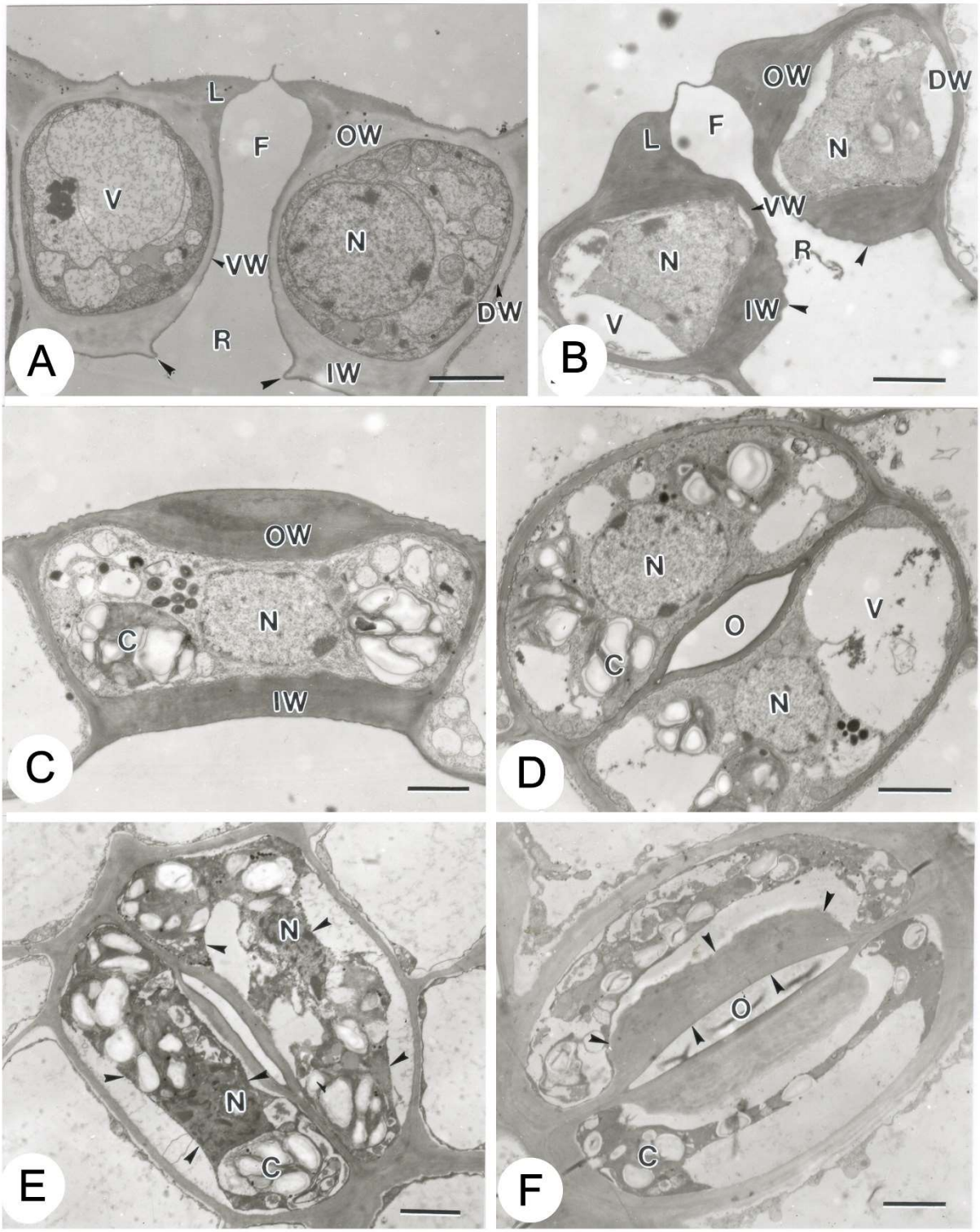

Fig. 2. TEM images of stomata from ***F. formosana*** f. ***shimadae***. **A**, Cross-section of stomata of a young leaf through the aperture. **B**, Cross-section of stomata at the mature leaf stage. **C**, Longitudinal section of guard cells at the mature stage. **D**, Paradermal section of stomata through the central region. **E**–**F**, Paradermal sections of stomata from leaf samples treated with 0.75 M mannitol solution. **E**, Paradermal section through the central region of stomata. Arrowheads indicate the plasma membrane of the plasmolytic cell. **F**, Paradermal section of stomata revealing guard cells near the substomatal chamber. Arrowheads indicate thickened cell wall around the pore. Scale bars: **A**–**F**=2 μm. DW, dorsal wall; L, Outer cuticular ridge; O, pore; N, nucleus; S, starch grain in chloroplast; V, vacuole; F, upper space; R, lower space; OW, external periclinal wall; IW, internal periclinal wall; VW, ventral wall.

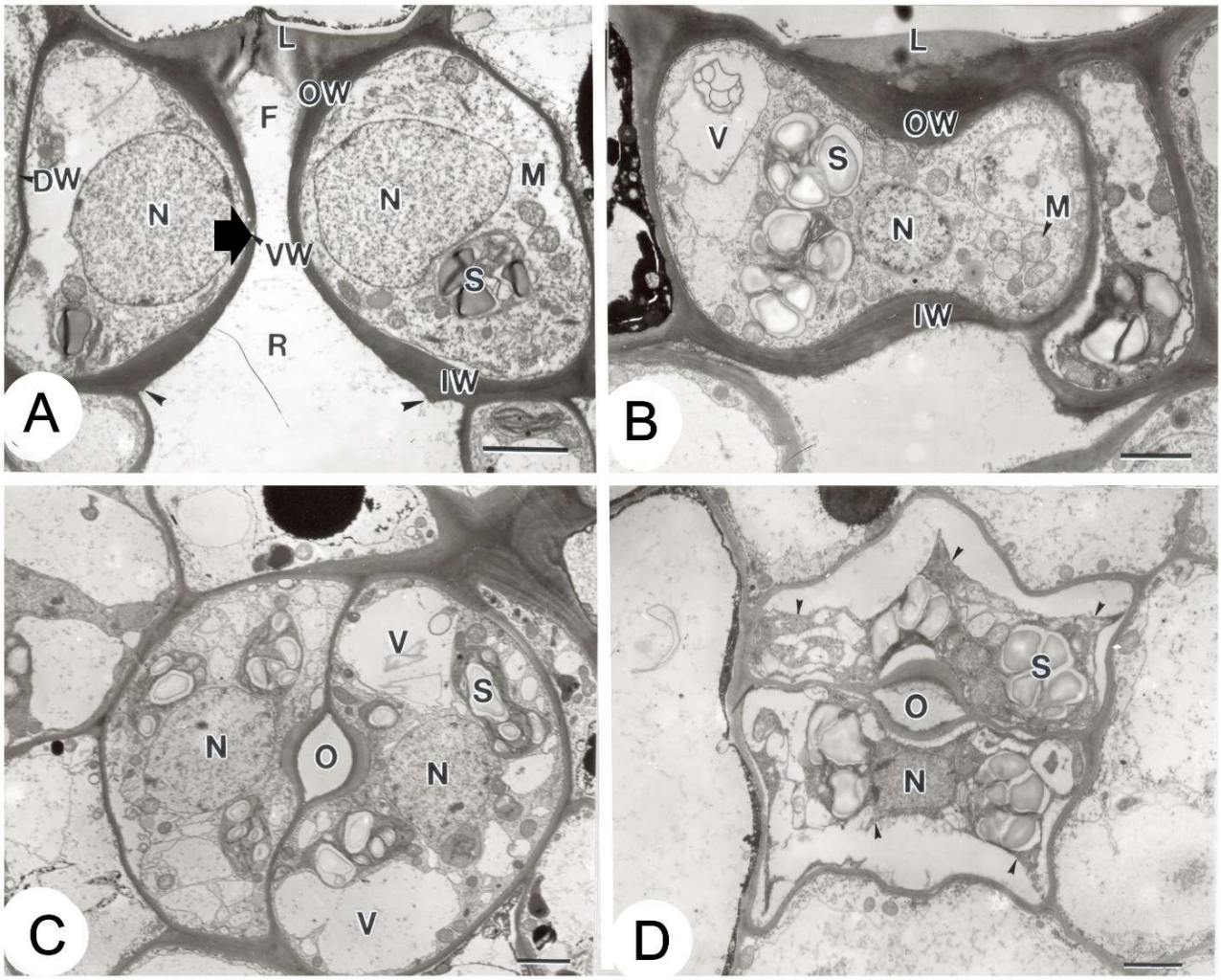

Fig. 3. TEM images of water pores of ***F. formosana*** f. ***shimadae*** hydathodes. **A,** Cross-section of water pore through its aperture. **B,** Cross-section of water pore along the longitudinal axes of its paired cells. **C,** Paradermal section of water pore through the central region. **D,** Paradermal section of a water pore from a leaf sample treated with 0.75 M mannitol solution. Scale bars: **A–D**=2 μm. DW, dorsal wall; M, mitochondrion; O, pore; N, nucleus; S, starch grain in chloroplast; V, vacuole; F, upper space; R, lower space; OW, external periclinal wall; IW, internal periclinal wall; VW, ventral wall.

where the aperture is defined by the outer cuticular ridges (Figs. 3A and 6H). During water pore development, starch-containing chloroplasts are present at all stages of the paired cells, but their thylakoid membranes do not form grana (i.e., unlike stomata) (Figs. 5E-F and 6D).

Physiological regulation of water pore and stomata apertures in response to light/dark, ABA, fusicoccin, or mannitol treatments

We subjected epidermal strips harboring water pores or stomata and incubated in the dark to light, 10 μM ABA, 1 μM fusicoccin, or 0.75 M mannitol treatments. As shown in Figure 7, stomatal guard cells treated with 10 μM ABA were significantly narrower than the control, and stomatal apertures significantly widened under lightor 1 μM fusicoccin treatment but narrowed under ABA treatment (Fig. 7A). In contrast, cell and pore widths for water pores did not change under any of these treatments (Fig. 7B). To assess the outcome of 0.75 M mannitol treatment under light, we stained treated epidermal strips with PI and observed them under confocal microscopy (Fig. 8). Autofluorescence from cell walls revealed that the stomatal apertures are almost completely closed in the dark or under 0.75 M mannitol+light treatment (Fig. 8A), but water pores remain open even under experimental treatments even though their outer cuticular ridges are tightly sealed (Fig. 8B). Notably, cell wall autofluorescence signal differed between stomata and water pores (arrows in Fig. 8), with strong fluorescent signals observed in the ventral portion and extremities of water pore cell walls, and whereas strong signal only appeared at the extremities of stomatal cell walls and ventral cell wall signal was weak.

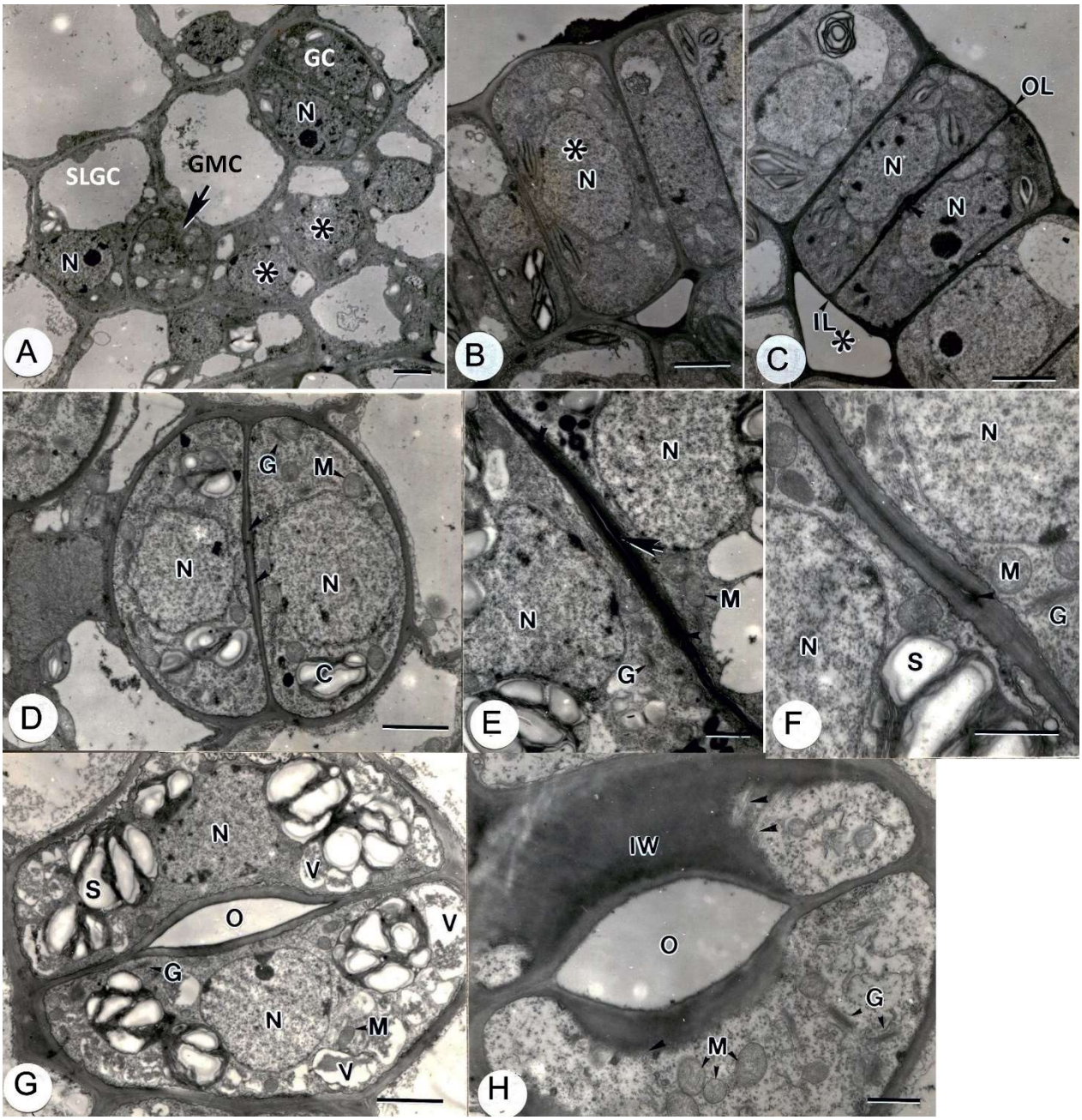

Fig. 4. TEM images showing ontogenesis, pore formation and wall-thickening of ***F. formosana*** f. ***shimadae*** stomata. **A,** Paradermal section of hypodermal cell layer at the young leaf stage. Asterisk indicates a stomatal lineage ground cell (SLGC) that may differentiate into pavement cells or may become meristemoid mother cells (MMCs), which later produce secondary meristemoids through asymmetric division. **B-C,** Cross-sections of the hypodermal layer of young leaves. **B,** Guard mother cell (asterisk). **C,** A pair of guard cells after a guard mother cell undergoes symmetric cell division. Asterisk indicates the sub-stomatal chamber. Arrow shows where lysis of the middle lamella occurs between cell walls of the guard cells. **D-F,** Paradermal sections of the hypodermal stomata of young leaves. **D,** Parademal section of stomata through the central region. Arrow shows where lysis of the middle lamella occurs between cell walls of the guard cells. **E-F,** Pore formation of stomata by lysis of the middle lamella, pore initiation (arrow in **E**) and pore lysis extension (arrowhead in **F**). **G-H**, Paradermal sections of stomata from mature leaves, showing the central region (**G**) and the area near the sub-stomatal cavity formed by a pair of guard cells (**H**). Arrows indicate microtubules radially orientated near the thickened cell wall. Scale bars: **A-D**=2 μm; **E-F**=1 μm; **G-H**=2 μm. G, Golgi body; GC, guard cell; GMC, guard-mother cell; OL, outer wall layer of guard cells; IL, inner wall layer of guard cells; M, mitochondrion; O, pore; N, nucleus; S, starch grain in chloroplast; V, vacuole; IW, internal periclinal wall.

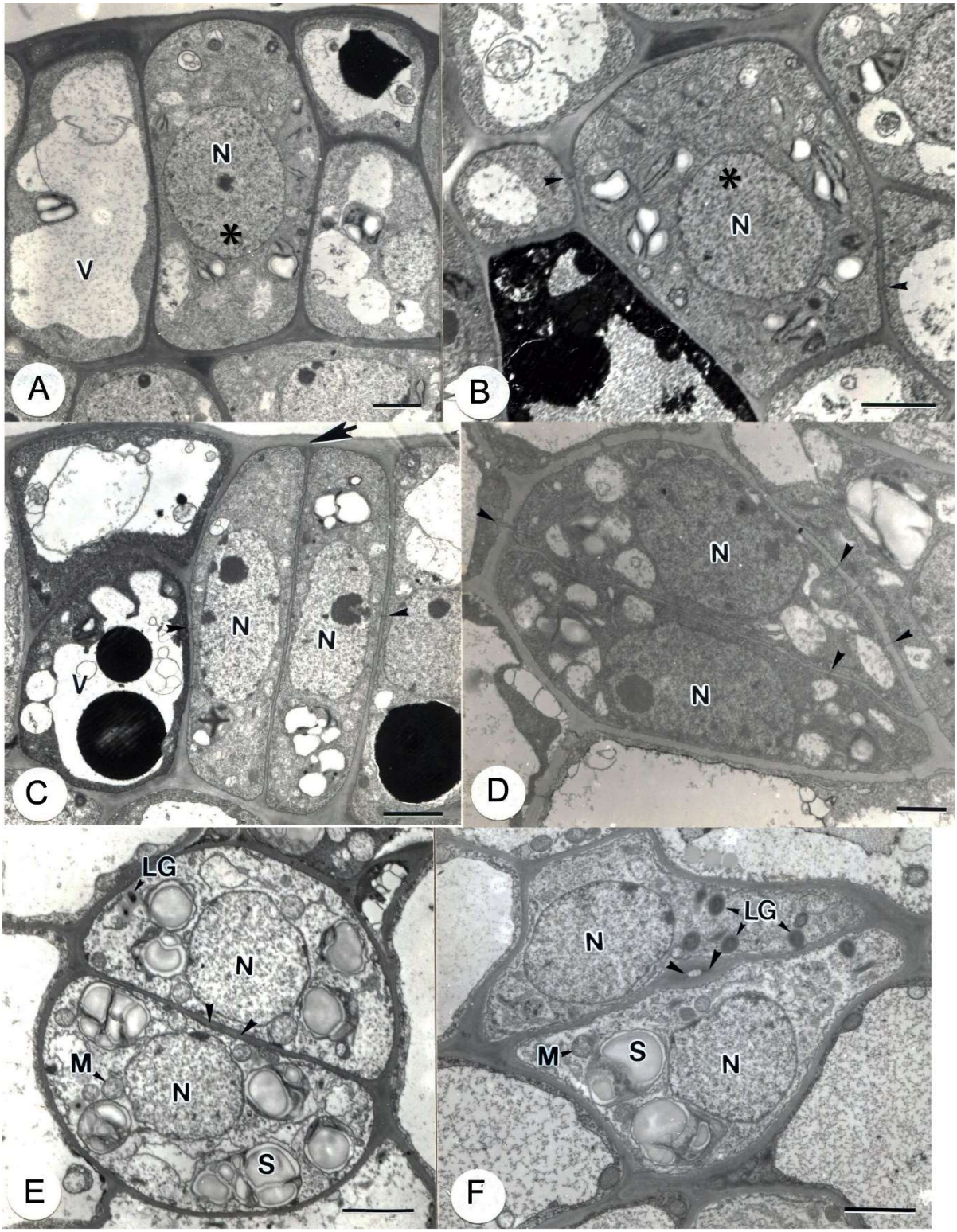

Fig. 5. TEM images showing ontogenesis of water pores from ***F. formosana*** f. ***shimadae*** hydathodes. **A-B,** Guard mother cells (asterisk) in cross-section (**A**) and paradermic sections (**B**). **C-D,** Guard mother cell transformation into guard cell pair by symmetric division. Cross-sections (**A** and **C**) and paradermal sections (**B** and **D)** of the surface layer of hydathodes. Outer cell walls exhibit partial thickening at the contact region between paired cells (**C**, arrow). Arrowheads indicate the plasmodesmta. **E-F**, Paradermal sections of water pores through the central region of young leaves. Lysis of the middle lamella between paired cells (**E**), and pore formation (**F**). Arrowheads show where lysis of the middle lamella occurs. Scale bars: **A-F**=2 μm. M, mitochondrion; LG, lipid granule; N, nucleus; S, starch grain in chloroplast; V, vacuole.

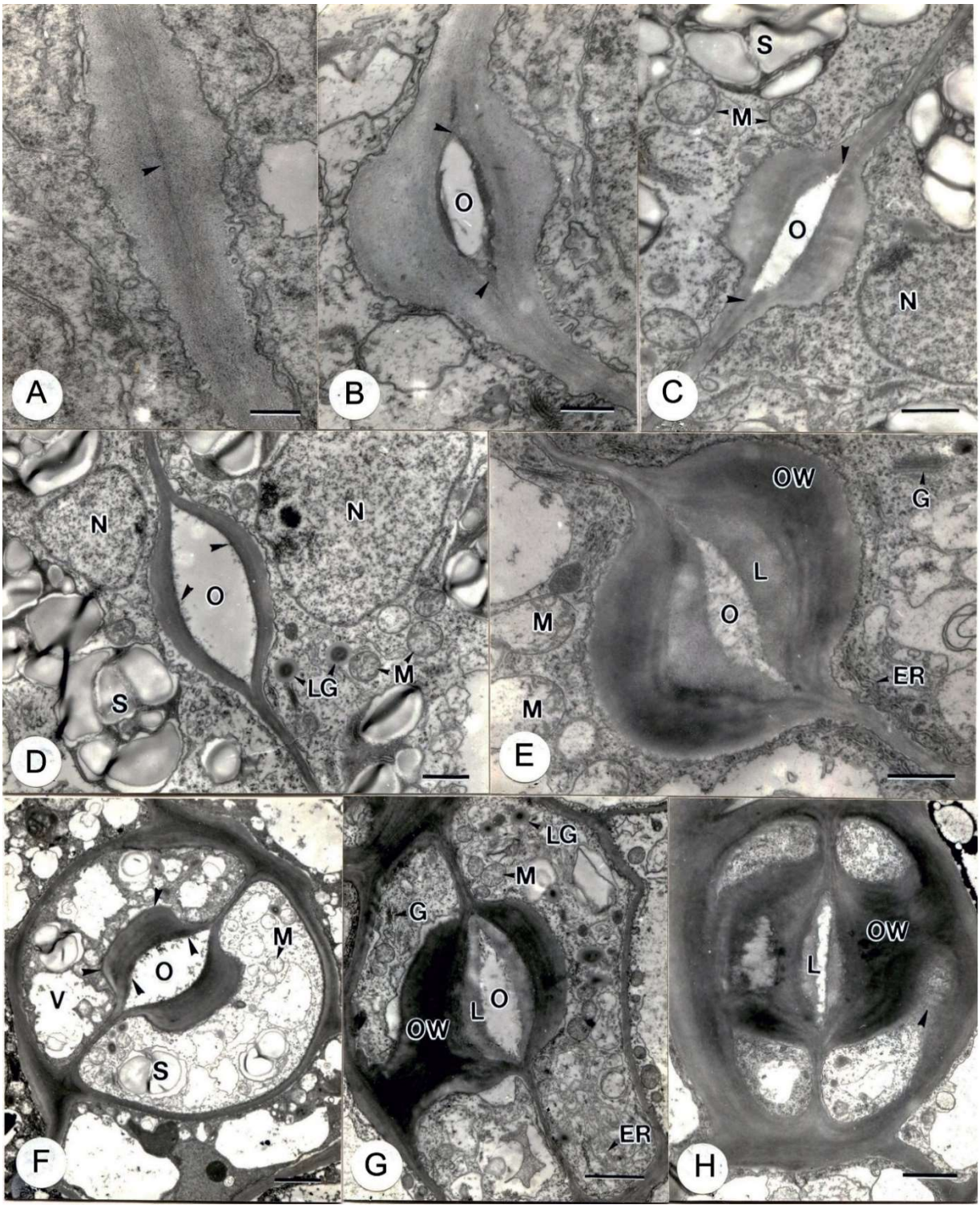

Fig. 6. TEM images of pore formation and water pore wall thickening from ***F. formosana*** f. ***shimadae*** hydathodes. **A-C**, Pore formation of water pores. Arrowheads show where lysis of the middle lamella occurs. **D-H,** Water pores at the mature stage. **D,** Paradermal section of water pore through the central region at the mature stage. Arrowheads indicate residues following middle lamella lysis. **E,** Paradermal section of water pore near pore opening. **F-H**, Cell wall thickening of water pores at different cross-sectional levels; the internal periclinal wall surrounding the pore (**F**), or the external periclinal wall extending to the outer cuticular ridge near the pore opening (**G and H**). Scale bars: **A**=0.25 μm, **B**=0.5 μm, **C-E**=1 μm**, F-H**=2 μm. ER, endoplasmic reticulum; G, Golgi body; L, Outer cuticular ridge; M, mitochondrion; LG, lipid granule; O, pore; N, nucleus; S, starch grain in chloroplast; V, vacuole; OW, external periclinal wall.

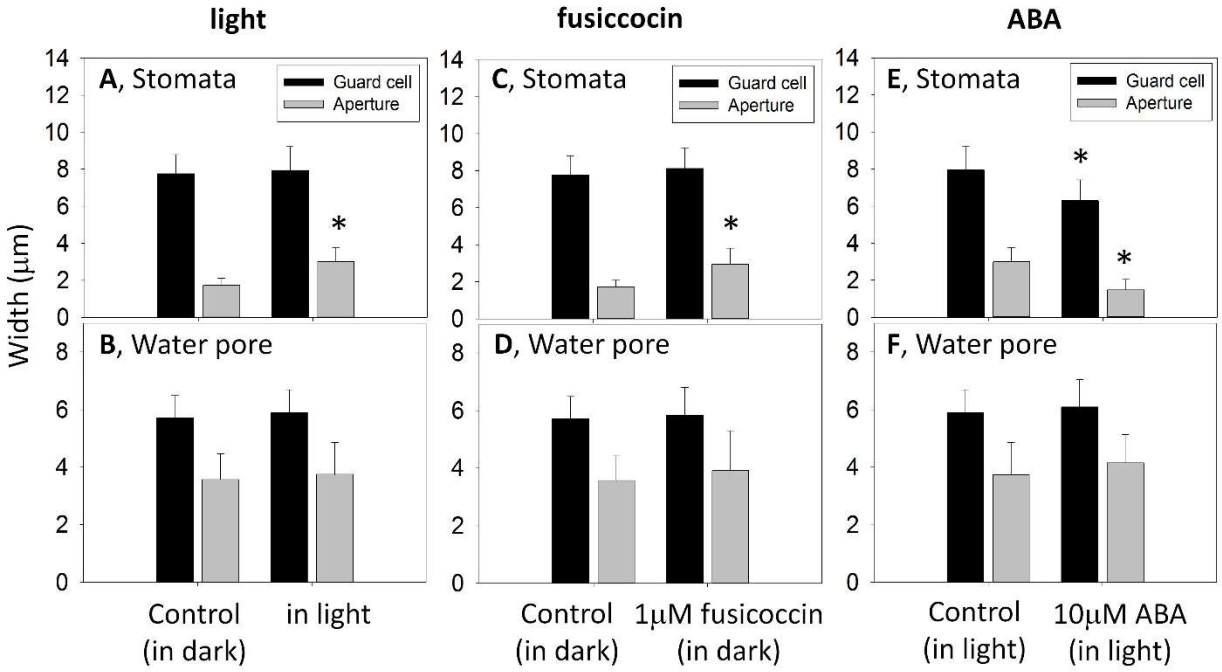

Fig. 7. Width of ***F. formosana*** f. ***shimadae*** water pore and stomatal cells and apertures in response to light, fusicoccin or ABA treatments. Leaf discs containing hydathodes were inoculated with phosphate buffer in the dark for 1 h as a control, before samples were subjected to light treatment for 1 h (**A-B)** or transferred to buffer containing 1 μM fusicoccin in the dark for 1 h (**C-D**) or buffer containing 10 μM ABA solution under light for 1 h (**E-F**). After treatments, the widths of apertures and guard/paired cells were measured under confocal microscopy. Experiments were repeated at least twice and asterisks (*) indicate significant differences between treatment samples and respective controls (Student's *t* test: * $p < 0.01$).

DISCUSSION

Ontogenetic analysis of water pores and stomata revealed that they exhibit similar early development but differ in how their respective pores/apertures are subsequently formed. Water pores are rigid structures that are permanently open and rounded in shape, which are unresponsive to the signals that normally induce stomatal closure. The pore of water pores is small, and the paired cells that form it are round or square. Does the existence of the water pore reflect the evolutionary transition of plants from aquatic to terrestrial? We believe that this morphology is related to continuous exposure to liquid water during differentiation. The water pores of aquatic plants are usually permanently opened that can support this opinion (Perdersen *et al.*, 1997). Pillitteri *et al.* (2008) reported that the water pores of hydathodes are evolutionarily related to stomata, and that their differentiation in Arabidopsis is regulated by MUTE (a basic helix-loop-helix protein that is required for differentiation of both stomata and hydathode water pores). That finding suggests that water pores and stomata may have a common developmental origin. The functions and ontogeny of water pores and stomata reflect their evolutionary roles. The structure and function of water pores evolved as plants transitioned from aquatic to terrestrial environments, and stomata have been proposed as one of the most important evolutionary adaptations allowing plants to colonize terrestrial habitats (Edwards *et al.*, 1998; Raven, 2002; Gray, 2007; Wang *et al.*, 2007).

Our observations reveal that *F. formosana* f. *shimadae* stomata are anomocytic (Fig. 1C), which has been reported for other species of *Ficus* such as *F. glomerata*, *F. benghalensis* and *F. carica* (Waman, 2015). As shown in Table 1, density of water pores is greater than stomatal density. It is well known that the stomatal density of developing leaves is regulated by environmental conditions such as light, carbon dioxide, humidity and temperature (Woodward, 1987; Croxdale, 2000; Kazama *et al.*, 2004; Casson and Gray, 2008; Casson and Hetherington, 2010; Pillitteri and Torii, 2012; Zheng *et al.*, 2013) and genetic signals (Yang and Sack, 1995; Berger and Altmann, 2000; Geisler *et al.*, 2000; Gray and Hetherington, 2004). Why are water pores more abundant than stomata in the *F. formosana* f. *shimadae* Firstly, stomatal density is responsive to changes in light intensity, with increased light intensity leading to an enhanced stomatal index in mature leaves (Schoch *et al.*, 1980; Thomas *et al.*, 2003; Coupe *et al.*, 2006). Since water pores are located on the upper leaf surface (unlike stomata on the lower surface) and consequently are subjected to higher light exposure than stomata, light may have resulted in the higher density phenotype. Secondly, *Arabidopsis thaliana* grown in closed containers exhibited increased stomatal density and

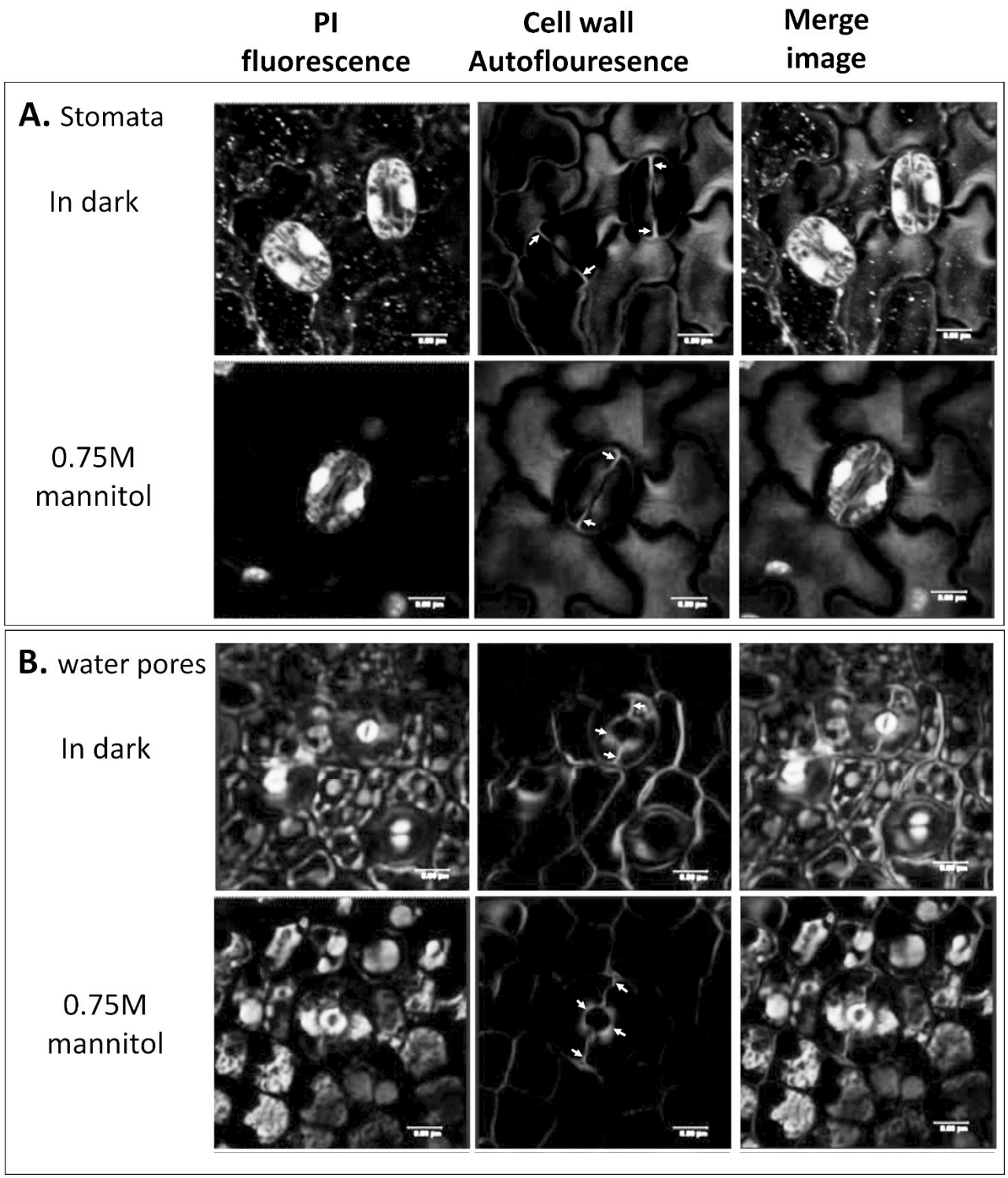

Fig. 8. Confocal microscopy of the stomata (**A**) and water pores (**B**) of ***F. formosana*** f. ***shimadae*** treated with 0.75 M mannitol and under dark conditions. Leaf discs were inoculated with phosphate buffer in the dark for 1 h, then transferred to buffer containing 0.75 M mannitol solution under light for 1 h. After treatment, leaf discs were inoculated with 10 μM PI solution and fluorescence of PI (excitation/emission maxima 535/617 nm), and cell wall autofluorescence (365/405 nm) was measured under confocal microscopy. Arrows indicate self-autofluorescence of water pore or stomatal cell walls.

clustering due to increased humidity or other variables that impeded transpiration (Lake and Woodward, 2008). We found that water pores were clustered on the surfaces of hydathodes (Fig. 1B), where guttation generates a highly humid environment. Thirdly, a constitutive triple reaction 1 (*ctr1*) mutant of *Arabidopsis* exhibits *thaliana* exhibits deficient ethylene signaling, with leaves of the *ctr1* mutant presenting increased stomatal density (Kieber *et al.*, 1993). Moreover, plants grown in the presence of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), also showed increased stomatal density and aggregation (Kieber *et al.*, 1993). Whether the high density and clustering of water pores is linked to salt and osmotic stress-induced ethylene biosynthesis requires further study.

We also observed multiple plasmodesmata linking the paired cells of water pores and their adjacent cells during development (Figs. 5C and 5D). *Arabidopsis* KOBITO1, a glycosyltransferase-like protein, is essential for both cellulose biosynthesis and regulation of the permeability of plasmodesmata. In a *kob1-3* mutant, increased permeability of intercellular plasmodesmata results in easy escape of the cell fate-specific factor *SPEECHLESS* (*SPCH*) from stomatal lineage cells, inducing stomatal clustering (Kong *et al.*, 2012). Similarly, *CHOR* encodes a putative callose synthase, *GLUCAN SYNTHASE-LIKE 8* (*GSL8*), which is required for callose deposition at the cell plate, cell wall, and plasmodesmata. Consistently, symplastic movement of fluorescent protein-tagged SPCH between epidermal cells was significantly increased in a *chor* mutant, again resulting in stomatal clustering (Guseman *et al.*, 2010). These mutagenic analyses may explain our observations of water pore clustering and the abundance of intercellular plasmodesmata during water pore development. Importantly, mature stomatal cells do not have functional plasmodesmata connections (Willmer & Sexton, 1979; Erwee *et al.*, 1985; Oparka and Roberts, 2001).

Our TEM analysis revealed that osmotic shock via 0.75 M mannitol treatment leads to stomatal closure, whereas it had no effect on water pores (Figs. 2F and 3D). In our physiological experiments, stomatal responses were observed to fusicoccin, ABA, osmotic shock, and illumination treatments. Stomata closed in the dark and under ABA and mannitol treatments (both latter two treatments under illumination), whereas fusicoccin (in the dark) or light treatments induced aperture opening (Figs. 7A and C). However, water pores remained permanently open under all of these treatments (Figs. 7B, D, F, and 8B). Previous studies have reported that the pore size of water pores is not regulated (Pillitteri and Dong, 2013; Hossain *et al.*, 2016). As reported for bryophytes (Brodribb and McAdam, 2011), we found that the pore size of water pores on the *F. formosana f. shimadae* hydathodes does not respond to light, ABA, fusicoccin or mannitol treatment.

We propose three anatomical features supporting the permanent opening of water pores. Firstly, pore length is less than one-third the length of the longitudinal axis of the water pore paired cells (Figs. 3C-D). Aylor *et al.* (1973) identified two structural features - the specific arrangement of cellulose microfibers and the limited length of protective cells - as being critical to the mechanism by which cells bend to open pores. In contrast, the long axes of stomatal openings tend to exceed half the length of the long axis of the guard cells (Esau, 1977), and ion channels regulated by various environmental factors can control cell expansion (Kollist *et al.*, 2014). However, since the ratio of pore length of cell length in water pores is small (Table I), this physical adjustment mechanism may not be operable. Secondly, we observed that the inner peripheral cell walls of water pores are notably thickened (Fig. 6F) and that the outer peripheral wall is connected to the outer cuticular ridge that forms the aperture (Figs. 6G-H). Finally, as shown in Fig. 8, autofluorescence signal from cell walls differed between water pores and stomata, implying that deposition of lignin is pronounced at the ventral and polar ends of water pores, but weak at the ventral walls of stomata. The markedly greater deposition of lignin in water pore cell walls may reduce their flexibility, keeping the pores permanently open.

Overall, our observations of the anatomical and morphological features of water pores and stomata, and our experimental analysis of responses to various treatments, reveal their evolutionary relationship in the *F. formosana* f. *shimadae* leaves is divergent. We suggest that further study of water pores offers an opportunity to understand how the epigenome is regulated at different stages of guard cell development and how functions other than those related to gas exchange and transpiration are obtained.

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